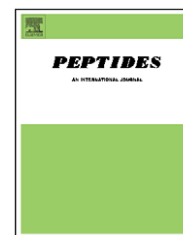


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Comparison of insect kinin analogs with cis-peptide bond, type VI-turn motifs identifies optimal stereochemistry for interaction with a recombinant arthropod kinin receptor from the southern cattle tick *Boophilus microplus*

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ABSTRACT

The multifunctional ‘insect kinins’ share the evolutionarily conserved C-terminal penta-peptide motif Phe-X¹-X²-Trp-Gly-NH₂, where X¹ = His, Asn, Ser, or Tyr and X² = Ser, Pro, or Ala; and are associated with the regulation of diuresis in a variety of species of insects. We previously reported the functional expression of a southern cattle tick (*Boophilus microplus*) G protein-coupled receptor that is activated by insect kinins. Four different stereochemical variants of each of the 4-aminopyroglutamic acid (APy) and tetrazole moieties, mimics of a cis-peptide bond, type VI β-turn in insect kinins were now evaluated on the expressed tick receptor using a calcium bioluminescence plate assay. This study represents the first investigation of the interaction of restricted-conformation analogs incorporating components that mimic specific conformations and/or peptide bond orientations in an expressed arthropod neuropeptide receptor. Analog Ac-RF[APy]WGa (2R,4S) was at least 10-fold more active than the other analogs, thus identifying the optimal stereochemistry for tick receptor interaction. The optimal stereochemistry for the tetrazole insect kinin analogs in the tick receptor assay was identified as (D,L). The APy is superior to the tetrazole as a scaffold for the design of mimetic insect kinin analogs. These biostable analogs provide new tools for arthropod endocrinologists and potential leads in the development of selective, environmentally friendly arthropod pest control agents capable of disrupting insect kinin regulated processes.

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1. Introduction

The insect kinins are multi-functional neuropeptides that were first isolated from the cockroach, *Leucophaea maderae*, in

which they stimulate hindgut contractions [7–9,26]. They have been isolated from a number of insect orders, including species of Dictyoptera, Lepidoptera, and Orthoptera. Shortly after their discovery, insect kinins from the cockroach were

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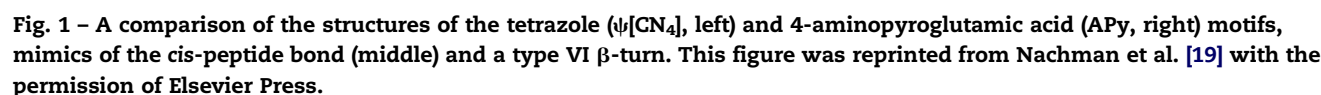
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Active cyclic analogs are of greater utility than linear analogs for defining the receptor-bound conformations because of their decreased conformational freedom [29]. Analysis of the conformations adopted by the head to tail, cyclic insect kinin analog cyclo(Ala-Phe-Phe-Pro-Trp-Gly), in which distance and angle constraints obtained from aqueous NMR spectra, were incorporated into molecular dynamics calculations, indicating the most prevalent conformation is the cisPro type VI β -turn over core residues 1–4 or Phe-Phe-Pro-Trp (see Fig. 1) [16,24,23,30,37]. Evaluation of analogs that

Prior to this investigation, no study has been undertaken to evaluate the interaction of restricted-conformation analogs of arthropod neuropeptides that incorporate components that mimic either a *cis* or *trans*-peptide bond, or a specific β -turn, on an expressed arthropod neuropeptide receptor. In this study, we evaluate four stereochemical variants each of two sets of analogs that contain either the 4-aminopyroglutamic acid (APy) and/or tetrazole moieties to identify the optimal stereochemistry for interaction with an expressed insect kinin receptor from the southern cattle tick, *Boophilus microplus* [10,11].

This work also leads to the identification of the optimal scaffold to design biostable, peptidomimetic agonist analogs as useful tools to arthropod endocrinologists and with the potential to disrupt critical processes in tick pests.

Insect kinin analogs were synthesized via Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Applied Biosystems, Foster City, CA) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) as previously described [13,19,21]. Crude products were purified on a Waters C₁₈ Sep Pak cartridge and a Delta Pak C₁₈ reverse-phase column (8 mm × 100 mm, 15 μm particle size and 100 Å pore size) on a Waters 600 HPLC as previously described [13,19,21].



2.2. Cell lines

Receptor cloning, transfection and selection of single clonal cell lines expressing the kinin (leucokinin-like) receptors from the southern cattle tick, *B. microplus* (AF228521) was reported previously [10,11]. The CHO-K1 cell line expressing the tick receptor, BmLK3 [10], was maintained in F-12K medium (Invitrogen) supplemented with 10% fetal bovine serum (EquiTech Bio, Kerrville, TX) with 400 µg/ml GENETICIN[®] at 37 °C and 5% CO₂.

2.3. Analysis of insect kinin peptide analogs activity by a Ca²⁺ bioluminescence plate assay

The functional analysis of ‘insect kinins’ with stably transformed CHO-K1 cells expressing the tick receptor [10] was by an intracellular calcium bioluminescence assay as described previously [28,35]. The assay uses aequorin, a photoprotein isolated from luminescent jellyfish (*Aequorea victoria*) and other marine organisms. Aequorin consists of a 189-amino acid polypeptide in a complex that includes a reactive group-coelenterazine, and oxygen. Upon addition of calcium ions, the photoprotein undergoes a conformational change. The filling of the calcium-binding sites on this protein results in the oxidation of bound coelenterazine using the protein-bound oxygen to form excited coelenteramide. When excited coelenteramide relaxes to the ground state it emits light at 469 nm [15].

Briefly, the aequorin plasmid mtAEQ/pcDNA1 (a kind gift from Drs. C. J. P. Grimmelikhuijzen and Michael Williamson, University of Copenhagen, Denmark) was grown in *Escherichia coli* cells MC1061/P3 (Invitrogen Co., CA) and was purified with a Qiaprep spin miniprep kit (Qiagen Inc., CA). Transient transfection with this plasmid was as described previously [34]. For this, the cells expressing the myokinin receptors were grown in F12K media containing 10% fetal bovine serum and 400 µg/ml GENETICIN[®] to about 90% confluency in T-25 flasks at 37 °C and 5% CO₂. Cells were trypsinized and 2 × 10⁵ cells in 2 ml of media were seeded in each well of 6-well tissue culture plates (Nunc Brand, NY). Cells were allowed to grow for 24 h in the incubator. For transfection of cells in each well of 6 well plate 96 µl of OPTI-MEM media (Invitrogen Co., CA) was mixed with 4 µl of the transfection reagent Eugene 6 (Roche Applied Science, IN) in a microfuge tube. The mixture was incubated for 5 min at room temperature after which 1 µg of aequorin/pcDNA1 plasmid DNA in 10 mM Tris buffer pH8.5 was added and incubated for another 15–20 min at room temperature. This mixture was added drop wise to each well of 6 well plate with gentle manual shaking, plates were then incubated for 4–6 h at 37 °C and 5% CO₂ and then the media was changed to F12K media containing 10% fetal bovine serum without antibiotic. After 24 h cells were trypsinized and transferred to 96-well, white, thin bottom micro titer plates (Costar 3610) at a density of 40,000 cells/100 µl per well and incubated for 24 h. To reconstitute the aequorin complex, cells were incubated in (90 µl/well) calcium-free DMEM media (GIBCO, Invitrogen Co., CA) containing 5 µM coelenterazine (Molecular Probes, Invitrogen Co., CA) for 3 h in the dark at 37 °C and 5% CO₂ [33]. Cells were then challenged with different concentrations of peptide analogs in a volume of 10 µl (10×) solubilized

in calcium-free DMEM media. In all the experiments C-terminal insect kinin core analog FFSWGa, was used as a positive control [10]. The assay was performed using the NOVOstar (BMG Labtechnologies) plate reader in bioluminescence mode at room temperature. Light emission (469 nm) was recorded every 2 s over a period of 50 s per well.

It should be noted that this bioluminescence assay performed on the NOVOstar plate reader is less sensitive [31,40] than other intracellular calcium measurement methods such as confocal fluorescent cytometry [10]. For instance the C-terminal insect kinin core analog FFSWGa shows an EC₅₀ of 8.4 nM in the fluorescence assay [10] and 570 nM in the NOVOstar bioluminescence assay, a difference of approximately 65-fold. Nonetheless, the NOVOstar bioluminescence assay was used in this study because it has the advantage of allowing measurements from a greater number of cells (40,000 cells/well), allows the use of DMSO and is a fast throughput assay. The fluorescence assay allows only measurements with a few cells at a time, it is time-consuming and thus, impractical when evaluating a series of analogs.

2.4. Agonist studies

The cells were challenged with various concentrations of analogs in a volume of 10 µl (10×) solubilized in calcium-free DMEM media. Each experiment was repeated independently on three different days with two replicates (wells) each day. Concentration–response curves were obtained by nonlinear regression curve fit analysis (sigmoidal dose–response equation with variable slope) using Prism software 4.0 (GraphPad, San Diego CA). Maximal bioluminescence responses from six individual replicates at each concentration were used for calculations of the EC₅₀'s [34].

3. Results

3.1. Effect of incorporation of aminopyroglutamate (APy) stereochemical variants on the activity of insect kinin C-terminal analogs

Four aminopyroglutamic acid stereochemical variants of the insect kinin pentapeptides FFSWGa and/or FFPWGa were tested on the tick kinin receptor stably expressed in CHO-K1 cells using a functional calcium bioluminescence assay. All the analogs were tested from 1 nM to 100 µM final concentrations. Three analogs 1518, Ac-RF[APy]WGa (2R,4S); RN2, Ac-RF[APy]WGa (2S,4S) and RN4, Ac-RF[APy]WGa (2R,4R) demonstrated agonist activity. The fourth analog RN3, Ac-RF[APy]WGa (2S,4R) failed to show any agonist activity even at high concentrations of up to 10 µM (Table 1, Fig. 2). The EC₅₀ values for analogs 1518, (2R,4S)-APy (1.56 µM); RN2 (2S,4S)-APy (11.08 µM), and the C-terminal insect kinin core analog FFSWGa (0.59 µM [35]) were statistically different (*P* < 0.05) (Fig. 2, Table 1). The observed maximal response for these analogs was high with 65% [(2R,4S)-APy], 48% [(2S,4S)-APy], and 45% [RN4, (2R,4R)-APy] of that of the positive control (Table 1). The (2R,4R)-APy analog was not potent at concentrations lower than 3 µM. It showed activity only at 10 µM and above, and therefore a dose–response curve could not be generated.

Table 1 – Estimated potencies ($EC_{50} \pm 95\%$ confidence intervals) and percentage of maximal bioluminescence response of different analogs in reference to FFSWGa tested on tick (BmLK3) receptor expressing cell line

Analog	Tick receptor (BmLK3 cell line)		Cricket diuretic assay [13]	
	$EC_{50} \pm CI$ (μM)	^a Maximal response (%)	Stimulation of Malpighian tubule fluid secretion EC_{50} (10^{-8} M)	Maximal response (%)
Amino pyroglutamate analogs				
1518, Ac-RF[APy]WGa (2R,4S)	1.56 ± 0.6	65.0 ± 7.0	0.7	93
RN2, Ac-RF[APy]WGa (2S,4S)	11.08 ± 3.6	48.0 ± 5.0	14	93
RN3, Ac-RF[APy]WGa (2S,4R)	N.D.	0	12	96
RN4, Ac-RF[APy]WGa (2R,4R)	N.D.	45.0 ± 3.0	7	83
Tetrazole analogs				
FF ψ [CN ₄][dA]WGa (<i>L,D</i>)	N.D.	0	(43) ^{b,c}	
FF ψ [CN ₄]AWGa (<i>L,L</i>)	N.D.	32 ± 6.0	34 ^b	100 ^b
F[dF] ψ [CN ₄]AWGa(<i>D,L</i>)	N.D.	47.0 ± 9.0	2 ^d	94
F[dF] ψ [CN ₄][dA]WGa (<i>D,D</i>)	N.D.	0	58 ^b	100 ^b
Positive controls				
FFSWGa	0.57 ± 0.07	100 ± 4.0		

EC_{50} are an estimate of the concentration required to induce a half-maximal response. The tetrazole analog *L,D* is an analogous stereochemical counterpart of 1518; *L,L* is an analogous stereochemical counterpart of RN2; *D,L* is an analogous stereochemical counterpart of RN3 and *D,D* is an analogous stereochemical counterpart of RN4, respectively.

^a Maximal response is the maximal bioluminescence response of each analog expressed as a percentage of the maximal response of the active core agonist FFSWGa (positive control) at $10 \mu M$. N.D.: The analog was tested but was either not very active or was not active at lower molarities, thus an EC_{50} could not be determined.

^b From Nachman et al. [18,22].

^c EC_{50} is for antagonism of native insect kinins.

^d Nachman et al. (unpublished data).

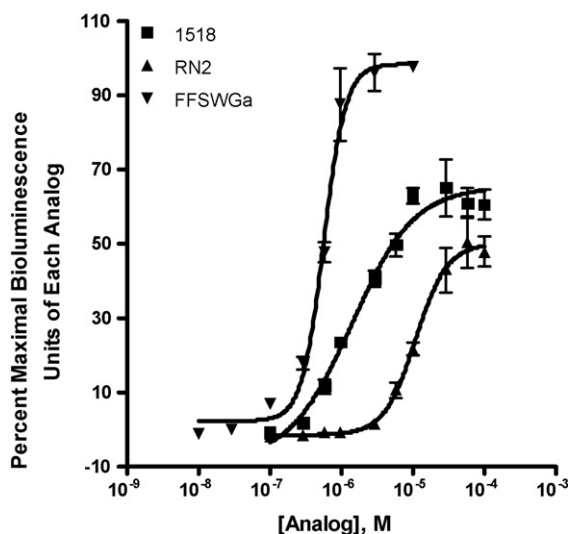


Fig. 2 – Activity of two stereochemical variants of insect kinin analogs that incorporate the cis-peptide bond, type VI-turn mimic APy on the tick kinin receptor. The analogs shown are 1518 [Ac-RF[APy]WGa (2R,4S)] and RN2 [Ac-RF[APy]WGa (2S,4S)] (Table 1). The y-axis represents bioluminescence units expressed as a percentage of the maximal response observed for the agonist FFSWGa, the insect kinin active core used as the positive control. Statistical analysis and graphs were generated with the GraphPad Prism 4.0 software. Those analogs that either do not reach a plateau or are not active even at $10 \mu M$ are not shown in this figure.

The diuretic activity of these APy-containing analogs was previously reported [13] and the EC_{50} values are also shown in the Table 1 for comparison with the bioluminescence responses on the expressed tick receptor.

3.2. Effect of incorporation of tetrazole stereochemical variants on the activity of insect kinin C-terminal analogs

Four tetrazole stereochemical variants of the insect pentapeptides FFSWGa and/or FFPWGa, FF ψ [CN₄][dA]WG (*L,D*); FF ψ [CN₄]AWG (*L,L*); F[dF] ψ [CN₄]AWG (*D,L*) and F[dF] ψ [CN₄][dA]WG (*D,D*) were tested on the tick kinin receptor that was stably expressed in CHO-K1 cells using a functional calcium bioluminescence assay. All the analogs were tested from 1 nM to $100 \mu M$ final concentrations. Of the four analogs, two analogs, (*D,L*) and (*L,L*), showed agonist activity, with a maximal response of 32 and 47% of that of the positive control FFSWGa, respectively (Table 1). As none of the analogs was active at concentrations lower than 3–6 μM their dose-response curves could not be generated.

The diuretic activity of three of the tetrazole containing analogs was also reported previously [13] and the EC_{50} values are also shown in the Table 1 for comparison with the bioluminescence responses on the expressed tick receptor. As a group, the tetrazole analogs demonstrated less activity than their APy analog counterparts.

4. Discussion

Peptide bonds can be oriented in either cis or trans forms, and this orientation can have profound consequences on the active conformation of biologically active peptides and on

their interaction with the receptor site [4,14,22,19,13]. Peptide bonds in natural, linear peptides form a dynamic equilibrium between the two orientations in aqueous solution, and generally do not adopt one over the other until they interact with the receptor surface. Analogs that incorporate restricted-conformation components that mimic one of the two orientations over the other can aid in the determination of the orientation and conformation adopted during successful receptor interaction [4,14,22,19,13]. Prior to this study, no investigation has been reported on the use of analogs containing *cis* or *trans*-peptide bond mimics, and/or mimics of specific β -turns, to determine preferred peptide bond orientation or the conformation during successful interaction with expressed neuropeptide receptors from arthropod sources.

Previous data obtained from NMR spectroscopic studies with the head-to-tail cyclic analog cyclo(Ala-Phe-Phe-Pro-Trp-Gly), active in an insect kinin cricket diuretic bioassay, indicated that two major turn conformations could be detected in aqueous solution. These were 1-4 *cis*Pro, type VI β -turn (60%) and 2-5 *trans*Pro type I β -turn (40%). Both the tetrazole [14,21,18,38,39] and APy [19,27,13] moieties mimic the *cis*-peptide bond type VI β -turn.

Previous studies have indicated that the restricted-conformation, tetrazole insect kinin analog Phe-Phe- ψ [CN₄]-Ala-Trp-Gly-NH₂ (*L,L*) and the 4-aminopyroglutamic acid (APy) analog RN2 Ac-Arg-Phe-(2S,4S)-APy-Trp-Gly-NH₂ (Fig. 1), analogous to the *L,L* stereochemical configuration of the tetrazole analog, demonstrate significant activity in a cricket diuretic assay (Table 1) [22,19]. The data from this assay therefore provide strong evidence for the active conformation of the insect kinins in the cricket diuretic assay. The insect kinin (2S,4S)-APy analog demonstrated an EC₅₀ of 14×10^{-8} M and a 93% maximal response [19] (Table 1), 2.5-fold more active than the (*L,L*)-tetrazole analog [22]. The maximal response was not significantly different from that of the tetrazole analog. In the cricket diuretic bioassay, the (2R,4R)- and (2S,4R)-APy analogs demonstrated EC₅₀ values (7 and 12×10^{-8} M, respectively) that were not statistically different from the parent (2S,4S)-APy analog, whereas the (2R,4S)-APy analog proved to be about 10-fold more potent (EC₅₀ = 0.7×10^{-8} M) (Table 1) [13]. A comparison among the analogous stereochemical variants of the restricted conformations, such as (2S,4S)- and (2R,4R)-APy, and (*L,L*)- and (*D,D*)-tetrazole, are not statistically different. The two tetrazole analogs are about 3-fold less potent than their APy counterparts. However, the relatively potent activity of the (2R,4S)-APy analog stands in stark contrast with the analogous (*L,D*)-tetrazole analog which demonstrates partial antagonism of the native insect kinins in the cricket diuretic bioassay [19]. The (*D,L*)-tetrazole analog, is the most active in this analog group, with an EC₅₀ of 2×10^{-8} M (maximal response: 94%) [Nachman et al., unpublished data].

Two APy analogs 1518, Ac-RF[APy]WGa (2R,4S) and RN2, Ac-RF[APy]WGa (2S,4S) elicited activity on the expressed tick kinin receptor of sufficient potency to allow the calculation of an EC₅₀ value (Table 1). The (2R,4S)-APy analog was also more active in the cricket diuretic assay (Table 1). The analog RN4, Ac-RF[APy]WGa (2R,4R) was strong enough to show some agonist effect on the receptor. Tetrazole analogs FF ψ [CN₄]AWGa (*L,L*) and F[dF] ψ [CN₄]AWGa (*D,L*) showed max-

imum responses of 32 and 47%, respectively, on the tick receptor. The activity of these APy and tetrazole analogs in the bioluminescence plate assay provides strong evidence that the active conformation of the insect kinins for interaction with the tick receptor is a 1–4 *cis*Pro, type VI β -turn, as it is for the insect diuretic assays.

The (2R,4S)-APy analog was the most potent analog by a factor of 10, in both the tick receptor and cricket diuretic assays. Despite the major modifications of the insect kinin structure, this mimetic analog is only 2.7-fold less potent in the tick receptor than its parent peptide, the insect kinin active core (Table 1). The 10-fold greater activity of the (2R,4S)-APy analog over the other three variants has been attributed in a previous study [13] to its greater flexibility, which would impart more freedom to adopt a better fit with the receptor site. The relatively potent activity of the (2R,4S)-APy analog in the bioluminescence and diuretic assays stands in stark contrast with the analogous (*L,D*)-tetrazole analog which demonstrates no activity and partial antagonism of the native insect kinins in the tick receptor and cricket diuretic assays, respectively. The (*D,L*)-tetrazole analog is the most active tetrazole analog in the tick receptor and the cricket diuretic assay (Table 1). The APy stereochemical counterpart [(2S,4R)-APy] is also active in the cricket diuretic bioassay, but completely inactive in the tick receptor assay. The (*D,D*)-tetrazole analog is completely inactive in the tick receptor assay, and its APy stereochemical counterpart [(2R,4R)-APy] demonstrate only weak activity. In contrast, these same two analogs demonstrate considerable activity in the cricket diuretic bioassay (Table 1). It is clear that the expressed tick receptor and the receptor associated with the insect kinin cricket diuretic assay feature both similarities and differences in their responses to the eight stereochemical analogs. For both assays, the APy is superior to the tetrazole moiety as a mimetic component in insect kinin core analogs. In addition, the optimal stereochemistry for the APy scaffold is (2R,4S)-APy for both the tick receptor and cricket diuretic assay systems. The optimal stereochemistry for the tetrazole group is (*D,L*) for both assay systems. However, while the (*L,D*)-tetrazole analog shows no agonist response in the tick receptor assay, it demonstrates partial antagonism of the natural insect kinins in the cricket diuretic bioassay. Otherwise, the cricket diuretic bioassay is clearly more tolerant to the changes in stereochemistry of the two *cis*-peptide bond mimetic components. This becomes evident in comparisons of the disparate activities of the (*L,D*)- and (*D,D*)-tetrazole analogs as well as the (2S,4R)-APy analogs in the two assay systems (Table 1).

It should be noted that the NOVOstar bioluminescence method used to evaluate the response of the expressed tick receptor to the 'insect kinin' analogs in this study is less sensitive as compared to the less practical fluorescence method that we have previously employed [10]. The bioluminescence plate assay is between 50 and 70-fold less sensitive (see Section 2). This difference should be taken into account when estimating the potency that these analogs would likely demonstrate in *in vitro* or *in vivo* physiological bioassays. An analog that is active at 1 μ M in the bioluminescence assay would likely be active at 20 nM or lower in physiological bioassays.

The linear insect kinins are peptidase susceptible and thus undergo rapid degradation by peptidases in the hemolymph [12]. The primary site of tissue-bound peptidase attack has been reported to be the amide bond between the Pro-Trp (or Ser-Trp) residues in the C-terminal pentapeptide core region [21]. The APy moiety precedes the Trp residue and would be expected to protect the adjacent amide bond from hydrolysis by peptidases.

In summary, the optimal stereochemistry for tick insect kinin receptor interaction has been identified as (2R,4S)-APy for the APy moiety and (D,L) for the tetrazole component, although the APy is clearly superior to the tetrazole moiety as a scaffold for the design of new biostable, bioavailable agonist analogs of this important class of arthropod neuropeptides. Although the physiological role(s) of the insect kinins in ticks is currently unknown, biostable analogs will provide important tools to arthropod endocrinologists in the quest to determine their function. Future research efforts will focus on the development of biostable, peptidomimetic analogs that can potentially disrupt the life processes regulated by the insect kinins, with the potential to provide new candidates as either research tools for neuroendocrinologists and/or future pest arthropod control agents.

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